Osteoarthritis and Cartilage 24 (2016) 146–157

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Ageing is associated with reduction of mechanically-induced activation of Smad2/3P signaling in articular cartilage

W. Madej † ‡ *, A. van Caam ‡, E.N. Blaney Davidson ‡, G. Hannink †, P. Buma †, P.M. van der Kraan ‡

† *Orthopaedic Research Laboratory, Radboudumc, Nijmegen, The Netherlands*
‡ *Experimental Rheumatology, Radboudumc, Nijmegen, The Netherlands*

ARTICLE INFO

Article history:
Received 26 March 2015
Accepted 27 July 2015

Keywords:
Cartilage
Ageing
TGF-beta
Smad2/3P signaling
Mechanosensitivity
Signal transduction

SUMMARY

Objective: Mechanical signals control key cellular processes in articular cartilage. Previously we have shown that mechanical compression is an important ALK5/Smad2/3P activator in cartilage explants. However, age-related changes in the cartilage are known to affect tissue mechanosensitivity and also ALK5/Smad2/3P signaling. We have investigated whether ageing of cartilage is associated with an altered response to mechanical compression.

Design: Articular cartilage explants of two different age groups (young-6–36 months old, aged-6 – 13 years old) were subjected to dynamic mechanical compression with 3 MPa (physiological) or 12 MPa (excessive) load. Subsequently, essential cartilage extracellular matrix (ECM) components and tissue growth factors gene expression was measured in young and aged cartilage by QPCR. Furthermore, the ability of young and aged cartilage, to activate the Smad2/3P signaling in response to compression was analyzed and compared. This was done by immunohistochemical (IH) Smad2P detection and Smad3-responsive gene expression analysis.

Results: Aged cartilage showed a highly reduced capacity for mechanically-mediated activation of Smad2/3P signaling when compared to young cartilage. Compression of aged cartilage, induced collagen type II (*Col2a1*) and fibronectin *(Fn1)* expression to a far lesser extent than in young cartilage. Additionally, in aged cartilage no mechanically mediated up-regulation of bone morphogenetic protein 2 (*Bmp2*) and connective tissue growth factor (*Ctgf*) was observed.

Conclusions: We identified age-related changes in cellular responses to mechanical stimulation of articular cartilage. We propose that these changes might be associated with age-related alterations in cartilage functioning and can underlie mechanisms for development of age-related cartilage diseases like osteoarthritis (OA).

Introduction

Mechanical signals have been shown to play a crucial role in cartilage formation as well as in tissue maintenance[1]. One of the major consequences of mechanical stimulation on cartilage is regulation of cartilage tissue matrix proteins expression, including aggrecan[2], collagen type II[3], fibronectin[4] and perlecan[5]. Furthermore in chondrocytes, the expression of many growth factors crucial for cartilage maintenance like transforming growth factor beta 1-*Tgfb1*[6] connective tissue growth factor-*Ctgf*[7] and bone morphogenetic protein 2-*Bmp2*[8] is modulated by mechanical signals. Additionally various of intracellular signaling cascades are also mechanosensitive, including Smad2/3P[6], FAK[9] and ERK[10].

Age-related changes in cartilage affect the extracellular matrix (ECM) as well as the chondrocytes. In articular cartilage the size, structure and sulfation characteristics of aggrecan in the ECM change during ageing[11,12]. Because aggrecan is the main determinant of the water content in cartilage ECM, changes in aggrecan result in reduction of tissue resiliency, hydratation and finally

* Address correspondence and reprint requests to: W. Madej, Radboud University Medical Center, 547 Orthopaedic Research Laboratory, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands. Tel: 31-(0)24-36-14930; Fax: 31-(0)24-35-40555.
E-mail addresses: Wojciech.Madej@radboudumc.nl (W. Madej), Arjan.vanCaam@radboudumc.nl (A. van Caam), Esmeralda.BlaneyDavidson@radboudumc.nl (E.N. Blaney Davidson), Gerjon.Hannink@radboudumc.nl (G. Hannink), Pieter.Buma@radboudumc.nl (P. Buma), Peter.vanderKraan@radboudumc.nl (P.M. van der Kraan).

http://dx.doi.org/10.1016/j.joca.2015.07.018
1063-4584/

volume. Furthermore, age-related glycation of collagens has been shown, causing increase in stiffness of the cartilage ECM[13,14]. In addition, chondrocytes are susceptible to senescence during ageing[15]. Importantly, many studies have shown an age-related declined responsiveness and/or disrupted signaling of key cartilage growth factors, including IGF1[16], BMP7[17] and TGFβ[18,19].

Particularly TGFβ is an essential anabolic growth factor in articular cartilage as it prevents deleterious chondrocyte terminal differentiation[20]. Importantly, TGFβ can signal via two different type I receptors-ALK-5 and ALK-1 in chondrocytes, being able to induce opposing effects in cartilage. There is evidence that TGFβ action in restriction of cartilage terminal differentiation is limited to TGFβ signaling via the type I receptor-TGFBR1 (ALK-5), followed by Smad2 and Smad3 phosphorylation[18,20]. Recently we showed that mechanical compression potently activates Smad2/3P signaling in young mature articular cartilage which was apparently TGFBR1 (ALK-5) controlled[6]. Independently, *in vivo* studies in mice demonstrated a strong reduction in ALK-5 expression in ageing articular cartilage[18,21].

Considering the fact that current understanding of mechanotransduction events is based on the studies of tissues from young experimental models, there is a need to investigate how age-related changes, in the cartilage ECM and cells, influence cellular response *in situ* to mechanical stimulation. Evidence for altered mechanosensitivity of articular cartilage in many aspects could provide more insight into understanding age-related articular cartilage disease like osteoarthritis (OA).

The purpose of this study was to investigate if aged articular cartilage, responds differently to dynamic mechanical compression than young cartilage. To investigate this, we analyzed the effect of physiological (3 MPa) and excessive (12 MPa) mechanical compression on the gross structural changes, expression of ECM components together with essential cartilage growth factors and activation of Smad2/3P signaling in young and aged articular cartilage.

Materials and methods

Articular cartilage explants culture

Bovine articular cartilage explants were harvested from metacarpophalangeal joints (MCP) of two different age groups (exact ages, established on original abattoir documentation are provided in figure legends of each experiment and in Supplementary Tables 1 and 2). Joints were obtained from the local abattoir within 3 h *post mortem*. Full cartilage thickness (young-986 ± 34 μm, aged-725 ± 42 μm thick) explants (without sub-chondral bone) were isolated with a 4 mm biopsy punch (Kai-medical, Japan). After isolation, all specimens were equilibrated for 48 h in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Gibco®, UK) (1 ml medium per one 4 mm Ø explant) with addition of Antibiotic-Antimycotic (contains 10,000 units/mL of penicillin, 10,000 μg/mL of streptomycin, and 25 μg/mL of Fungizone®) (Gibco®, USA) in standard culture conditions (37°C, 5% CO_2 and 95% humidity). No serum was added to the medium.

Histological analysis

Full thickness osteochondral biopsies were isolated from MCP, with a diamond coated blade saw. Biopsies were fixed overnight at 4°C in 10% phosphate buffered formalin (pH 7) and decalcified for 1 week in 10% formic acid at room temperature. Specimens were dehydrated with a tissue processing apparatus (Pathos, Milestone Medical Inc.). After embedding in paraffin, sections of 7 μm were cut, than stained with Mayer's hematoxylin and visualized under a standard light microscope.

Dynamic mechanical compression of cartilage explants

Explant from stimulation groups were subjected to force controlled, sinusoidal, unconfined, dynamic mechanical compression with 3 or 12 MPa for 30 min, with 1 Hz, exactly like described before[6]. Full detailed description is included in Supplementary materials.

Articular cartilage strain monitoring during dynamic mechanical compression

The displacement of cartilage explants was monitored during the complete duration of dynamic, force controlled mechanical compression with 3 MPa or 12 MPa pressure. Compressions were performed as described before[6]. Data were recorded with WinTest® software (BOSE ElectroForce, USA). Displacement values were corrected for the thickness of the specimen (measured on histological section of unloaded controls) to calculate strain values.

Total mRNA isolation and quantitative RT-PCR (Q-PCR)

mRNA isolation and Q-PCR were performed exactly like described before[6]. Full detailed description is included in Supplementary materials. Primers used are included in Table I.

Table I
Primers list. Only primers with efficiency between 93% and 105% were used

Gene symbol	Full gene name	Ref seq	Product length	Efficiency	Forward 5′ → 3′	Reverse 5′ → 3′
bGapdh	glyceraldehyde 3-phosphate dehydrogenase	NM_001034034.2	90	100.92%	CACCCACGGCAAGTTCAAC	TCTCGCTCCTGGAAGATGGT
bAcan	aggrecan	NM_173981.2	144	96.84%	TGAAACCACCTCCACCTTCCATGA	TCAAAGGCAGTGGTTGACTCTCCA
bBmp2	bone morphogenetic protein 2	NM_001099141.1	73	105.35%	CGCAGCTTCCATCACGAA	AGAAGAATCGCCGGGTTGTT
bCol2a1	collagen, type II, alpha 1	NM_001001135.2	60	96.84%	TGATCGAGTACCGGTCACAGAA	CCATGGGTGCAATGTCAATG
bCtgf	connective tissue growth factor	NM_174030.2	86	105.35%	GACTTCGGCTCCCCAACCAA	TGGTACACAGTTCCTCCGAAAAT
bFn1	fibronectin 1	XM_005202786.1	95	96.84%	GCACCACTCCCGACATTACT	CTGATCGGCATGGACCACTT
bHspg2	heparan sulfate proteoglycan 2	XM_005197610.1	160	105.35%	GGGACTTCCAGATGGTTTATTTC	TGGTCTCCAGGGATCTTCA
bJunb	jun B proto-oncogene	NM_001075656.1	139	96.84%	CCTTCTACCACGACGACTCA	CCGGGTGCTTTGAGATTTCG
bSerpine1	plasminogen activator inhibitor type 1	NM_174137.2	55	100.92%	CGAGCCAGGCGGACTTC	TGCGACACGTACAGAAACTCTTGA
bSmad7	SMAD family member 7	NM_001192865.1	72	105.35%	GGGCTTTCAGATTCCCAACTT	CTCCCAGTATGCCACCACG
bTgfb1	transforming growth factor, beta 1	NM_001166068.1	80	105.35%	GGTGGAATACGGCAACAAAATCT	GCTCGGACGTGTTGAAGAAC
bTgfbr1	transforming growth factor, beta receptor 1	NM_174621.2	75	93.07%	CAGGACCACTGCAATAAAATAGAACTT	TGCCAGTTCAACAGGACCAA

Immunohistochemical (IH) analysis

At 2 h after compression, samples were fixed over night at 4°C in 10% phosphate buffered formalin. Specimens were dehydrated and embedded in paraffin. 7 μm thick sections were cut and mounted on Superfrost™ Plus Microscope Slides (Thermo Scientific, USA). Then, the immunohistochemistry for c-terminally phosphorylated SMAD2P (rabbit mAb anti Phospho-Smad2 (Ser465/467) (1:100) (Cell Signalling Technology, Danvers, Massachusetts, USA)) was performed as previously described[21].

Computational scoring of Smad2P IH

To score the load-induced Smad2P staining, first the detection threshold was set to detect only intense Smad2P staining. Obtained values of positive Smad2P staining were first corrected for ROI (region of interest). Then values of each experimental group were corrected for values of staining in unloaded controls (to show the load-induced Smad2P in each age). Finally, the load-induced Smad2P staining was corrected to the average cell number in a certain age group.

To score the nuclear localization of Smad2P staining, first the threshold was set to detect only Smad2P nuclear staining. In each scored section it was verified that staining detected after thresholding was always located in the cell nucleus but not in the cytoplasm. Obtained values were corrected for ROI (region of interest), and then corrected for average cell number in the certain age group.

All scoring values were expressed as a % of young. Scoring was performed with LAS (Leica Application Suite, Leica Microsystems, Germany).

Statistical analysis

Quantitative data of gene expression analysis were expressed as a grouped column scatter of multiple repeats with displayed mean. All experiments were repeated 5 times on material isolated from different animals, $N = 5$ (experimental setups are included in Supplementary Tables 1 and 2). First all datasets were checked for normality using the Shapiro–Wilk test. Linear mixed models were used to estimate the effects of compression level and age on gene expression. Linear mixed models take into account the correlated nature of repeated measures on cartilage isolated from the same subject. All the analyses were performed with the statistical software packages: SPSS 20.0 (SPSS, Chicago, USA).

Additionally, linear mixed models were used to fit the individual strain profiles of dynamically compressed cartilage explants of different ages (Supplementary Table 2). The dependent variable was strain. The independent variables were the loading condition and age. Interaction terms between loading condition and age were included in the model. The intercept and the regression coefficients of time were treated as random effects. The estimated regression parameters with standard errors were used to calculate the mean strain profiles with 95% confidence intervals for each loading/age condition. These statistical analyses were performed using R version 3.1.2 with package 'nlme' (R Development Core Team).

Results

Age-related changes in articular cartilage structure

Histological comparison of articular cartilage of 2 year and 13 year old revealed a very prominent age-related reduction in cartilage thickness (Fig. 1). In young cartilage an irregular transition of cartilage to bone, active remodeling and abundant vascularity was observed. In contrast, in aged cartilage the transition to the subchondral bone was straight and a clear tide mark was present (Fig. 1). Furthermore, a significantly reduced number of chondrocytes was present in aged when compared to young cartilage ($P = 0.0002$) (Fig. 1 and Supplementary Fig. 2).

Articular cartilage of different ages responds differently to dynamic mechanical compression

Histological evaluation showed that 3 MPa mechanical compression did not cause any severe structural changes in 1 year or 10 years old cartilage (Fig. 2B and E, respectively) when compared to corresponding unloaded controls (Fig. 2A and D, respectively). In both cases, intact surfaces, normal matrix architecture, no enlargement/distortion of chondrons were observed. However, the disappearance of the unstained superficial cartilage zone caused by 3 MPa compression was noticed. 12 MPa compression did not result in severe structural changes of 1 year old cartilage either (Fig. 2C). However, in 10 years old cartilage, 12 MPa mechanical compression induced surface discontinuities in a form of vertical fissures (Fig. 2F). Examination of the sections under polarized light confirmed that no vertical fissures were present in the unloaded control (Fig. 2G) and 3 MPa (Fig. 2H) compressed cartilage but in 12 MPa compressed 10 years old cartilage vertical fissures penetrated the mid-zone (Fig. 2I).

Cartilage deformation as a function of time can deliver information about mechanical properties and water content of the tissue[22]. We analyzed load-induced cartilage deformation to compare young and aged cartage mechanical properties. Individual strain (deformation) profiles showed typical patterns over time, starting with a steep increase in strain followed by a gradual increase and flattening of the strain profile (Fig. 2J). Analysis of strain profiles showed that young cartilage was able to deform significantly more, starting from 1550 second ($P = 0.04$) compared to aged cartilage when subjected to 3 MPa mechanical compression (Fig. 2J). Comparison of cartilage deformation during 12 MPa mechanical compression revealed no significant differences in the amount of the deformation between young and aged cartilage (Fig. 2J).

Ageing affects the influence of dynamic mechanical compression on the expression of articular cartilage ECM components

Expression of cartilage ECM components is highly sensitive to mechanical signals[3]. We measured the impact of compression on expression of ECM components in young and aged cartilage. Dynamic mechanical compression had no effect on the expression of *bAcan (Aggrecan)* (Fig. 3A). However, at 2 h after compression a significantly diverse (3.9-fold $2^{2.0Ct}$, $P = 0.048$) regulation of *bCol2a1* expression with up-regulation in young and down-regulation in aged in cartilage by 12 MPa compression was found (Fig. 3B). Analysis of the *bFn1* expression at 6 h time point showed a significantly different regulation of *bFn1* expression by 12 MPa compression in cartilage of different age (Fig. 3C) (2.1-fold, $2^{1.1Ct}$, $P = 0.001$), with up-regulation in young cartilage and down-regulation in aged cartilage. Remarkably, at the 6 h time point, in both stimulation groups, a down-regulation of perlecan *(bHspg2)* was observed in aged cartilage whereas no changes in *bHspg2* expression were observed in young cartilage (Fig. 3D). This resulted in significantly different changes in expression levels of *bHspg2* in cartilage of different age (3.4-fold, $2^{1.8Ct}$, $P = 0.002$ for 3 MPa and 2.3-fold, $2^{1.2Ct}$ $P = 0.025$ for 12 MPa compressed cartilage) (Fig. 3D).

Ageing affects mechanically mediated expression of essential cartilage growth factors

Expression of many key cartilage growth factors, same like ECM components, is also mechanosensitive[6–8]. We studied if aged

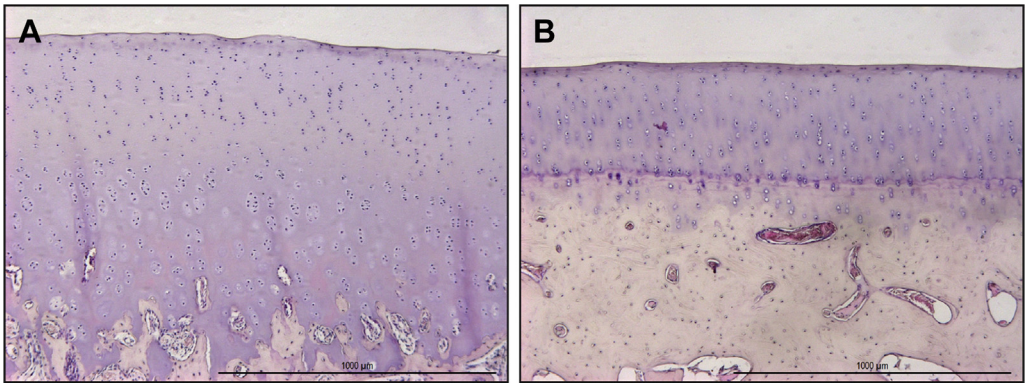

Fig. 1. Age-related changes in articular cartilage structure. Representative full thickness cross section of bovine articular cartilage with subchondral bone of a 2 years old individual (A) and of 13 years old individual (B).

articular cartilage has an altered mechanically-mediated regulation of key tissue growth factors.

At 2 h time point, a potent and significant ($P = 0.001$ for all cases) up-regulation of *bTgfb1* expression was measured in both age and stimulation groups (Fig. 4A). At 6 h time point, in 12 MPa compressed cartilage up-regulation was significant for both age groups ($P < 0.0001$ for young cartilage and $P = 0.001$ for aged cartilage) (Fig. 4A). At 2 h after compression, a down-regulation of *bCtgf* in aged cartilage compressed with 12 MPa ($P = 0.027$) was observed. This down-regulation was significant when compared to young cartilage compressed with 12 MPa (6.3-fold, $2^{2.7Ct}$ $P = 0.003$) (Fig. 4B). At 6 h time point, in both stimulation groups, an up-regulation of *bCtgf* was observed in young cartilage with down-regulation in aged cartilage. At this time point, a significantly opposed effect of mechanical compression on *bCtgf* expression regulation (10-fold, $2^{3.3Ct}$ $P < 0.0001$ for 3 MPa and 48.1-fold, $2^{5.6Ct}$ $P < 0.0001$ for 12 MPa compressed group) was identified in cartilage of different age. At 2 h time point, a pronounced up-regulation of *bBmp2* induced by both compression levels ($P < 0.0001$ for both stimulation groups) was seen, however only in young cartilage (Fig. 4C). Remarkably, in aged cartilage *bBmp2* was not affected by any level of mechanical compression (Fig. 4C). This revealed significantly different regulation of *bBmp2* expression by compression between young and aged cartilage compressed with 3 MPa (3.0-fold, $2^{1.6Ct}$, $P = 0.045$) and 12 MPa (4.2-fold, $2^{2.1Ct}$, $P = 0.011$) (Fig. 4C). The up-regulation of *bBmp2* expression in young cartilage was still present at 6 h after the compression in both stimulation groups ($P = 0.002$ for 3 MPa and $P < 0.0001$ for 12 MPa) whereas in aged cartilage still no regulation of *bBmp2* was observed. Thus, significantly different regulation of *bBmp2* in cartilage of different age was present in 3 MPa (2.2-fold, $2^{1.1Ct}$, $P = 0.048$) and in 12 MPa group (5.2-fold, $2^{2.4Ct}$, $P < 0.0001$).

Ageing reduces mechanically mediated phosphorylation of Smad2 in articular cartilage

Previously we showed that mechanical compression can act as a significant inducer of Smad2/3P signaling in mature articular cartilage[6]. Here, we analyzed if ageing affects mechanically-mediated phosphorylation of Smad2 in articular cartilage.

A clear induction of Smad2P staining was observed in young cartilage compressed with 3 MPa (Fig. 5Ab, e, h) when compared to unloaded control (Fig. 5Aa, d, g). Evident induction of Smad2P staining was also observed in 12 MPa compressed young cartilage (Fig. 5Ac, f, i) when compared to unloaded control (Fig. 5Aa, d, g). A reduced level of Smad2P staining in aged compressed cartilage was detected when compared to young cartilage. This was visible in 3 MPa (Fig. 5Ak, n) and 12 MPa (Fig. 5Al, o) compressed aged cartilage as well as in unloaded control (Fig. 5Aj, m). Computational scoring of Smad2P IH confirmed these observations (Fig. 5B). Aged cartilage showed highly reduced load-induced Smad2P staining when compared to young cartilage. This was the case for 3 MPa ($P < 0.0001$) as well as for 12 MPa compressed cartilage ($P < 0.0001$) (Fig. 5B).

Furthermore, prominent differences between dynamically compressed young and aged cartilage were observed in the localization of Smad2P staining. In young cartilage compressed with 3 MPa as well as with 12 MPa, Smad2P staining was mainly localized in the cell nuclei (Fig. 5Ca, b) whereas in aged cartilage Smad2P staining was more predominantly present in the chondrocyte cytoplasm (Fig. 5Cc, d). Computational scoring of Smad2P nuclear staining showed that in aged cartilage there is a significant reduction of nuclear Smad2P localization when compared to young cartilage ($P < 0.0001$). This was the case for both loading conditions (Fig. 5D).

Ageing reduces mechanically-induced activation of Smad3P signaling reporter genes

To investigate if the reduction of mechanically-induced TGFβ signaling in aged cartilage, indicated by the reduced Smad2P, is reflected in gene expression, the expression of downstream reporter genes for Smad3P in compressed cartilage isolated from individuals of different age was examined. These included-*Serpine1* (*Pai1*), *JunB* and *Smad7*. All of these genes contain a Smad Binding Element in their promoter[23–25].

Because in cartilage, TGFBR1 (ALK-5) is the main receptor activating Smad2/3 signaling pathway, the influence of age on basal expression of *bTgfbr1 (bAlk5)* in bovine cartilage was analyzed. Comparison of *bTgfbr1* expression levels between young and aged cartilage demonstrated significantly lower (2.4-fold, $2^{1.3Ct}$ $P = 0.002$) *bTgfbr1* expression levels in aged cartilage than in young cartilage (Fig. 6A).

At 2 h after compression a profound up-regulation of *bSerpine1* expression was observed, especially in young cartilage (Fig. 6B); 3 MPa mechanical compression of young cartilage up-regulated *bSerpine1* expression (32-fold, $2^{5\ Ct}$ $P < 0.0001$) whereas the same compression level in aged cartilage did not up-regulate *bSerpine1* expression significantly (Fig. 6B). Therefore, *bSerpine1* expression was significantly different up-regulated by 3 MPa compression in cartilage of different ages (6.1-fold, $2^{2.6Ct}$ $P = 0.002$) (Fig. 6B). In 12 MPa compression group, a significantly higher *bSerpine1* up-regulation was observed in young compared to aged cartilage (3.7-fold, $2^{1.9Ct}$ $P = 0.015$) (Fig. 6B). At 6 h after compression

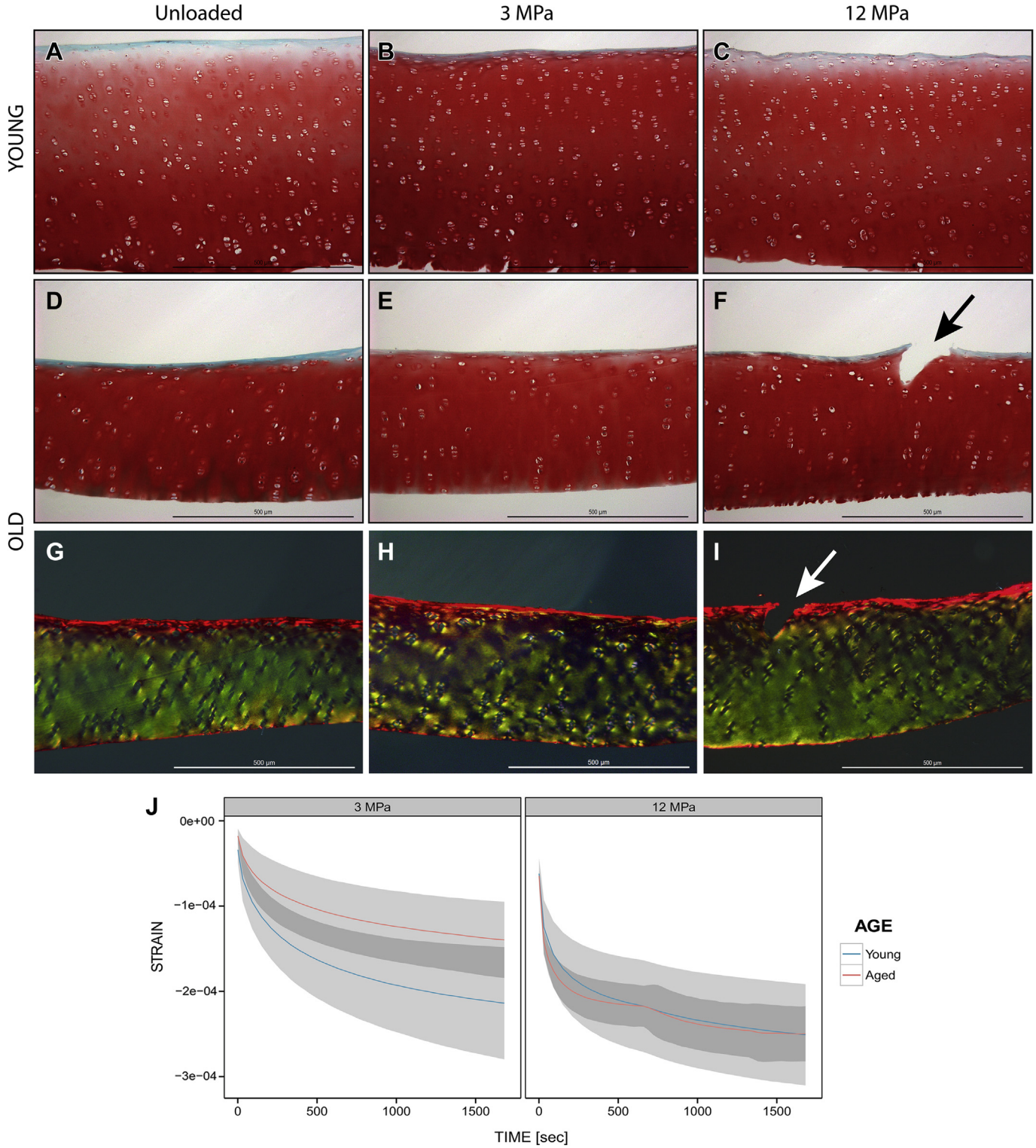

Fig. 2. Effect of dynamic mechanical compression on young and old articular cartilage. (A–F) Representative cross sections of articular cartilage stained with Safranin O and Fast Green. Intact articular cartilage specimens were dynamically compressed with 3 MPa or 12 MPa. Compression was performed as a sine wave with frequency of 1 Hz for 30 min (1800 cycles). (A, B, C) 1 year old cartilage: (A) unloaded, (B) 3 MPa compressed and (C) 12 MPa compressed cartilage, (D, E, F) 10 years old cartilage: (D) Unloaded, (E) 3 MPa compressed and (F) 12 MPa compressed cartilage. (G,H,I) Cross section of 10 years old cartilage stained with Picro Sirius Red and examined under the polarized light: (G) Unloaded, (H) 3 MPa compressed and (I) 12 MPa compressed cartilage. Arrows indicate the cartilage surface discontinuity. (J) Articular cartilage strain during dynamic mechanical compression with 3 or 12 MPa. Blue lines represent averaged strain of cartilage isolated from 1, 2 and 3 years old individuals. Red lines represent averaged strain of cartilage isolated from 6 and 10 years old individuals. Grey shadows represent the curve's 95% CI.

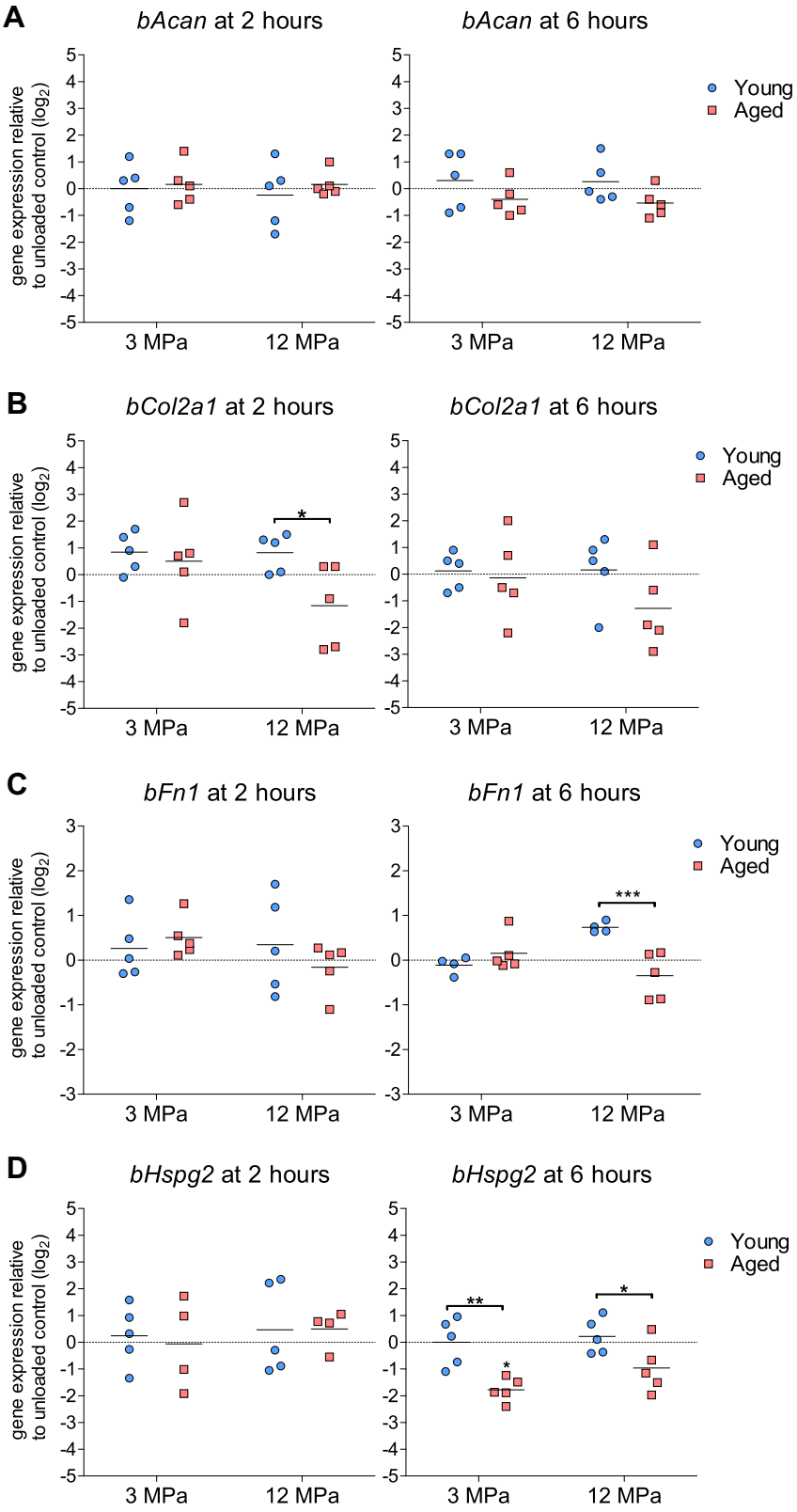

Fig. 3. Influence of dynamic mechanical compression on the expression of cartilage ECM components in cartilage of different age. Relative expression of bAcan (A), bCol2a1 (B), bFn1 (C) and bHspg2 (D) in young and aged cartilage. 12 MPa dynamic mechanical compression induced significantly different regulation of bCol2a1 (B), and bFn1 (C) expression in cartilage of different age. Both levels of mechanical compression down-regulated bHspg2 only in aged cartilage (D). Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal). Age of cartilage was as follows: Young-three 7 months, and two 11 months old; aged: 12, 10, 8 and two 9 years old. *-$P \leq 0.05$; **- $P \leq 0.01$; ***- $P \leq 0.001$.

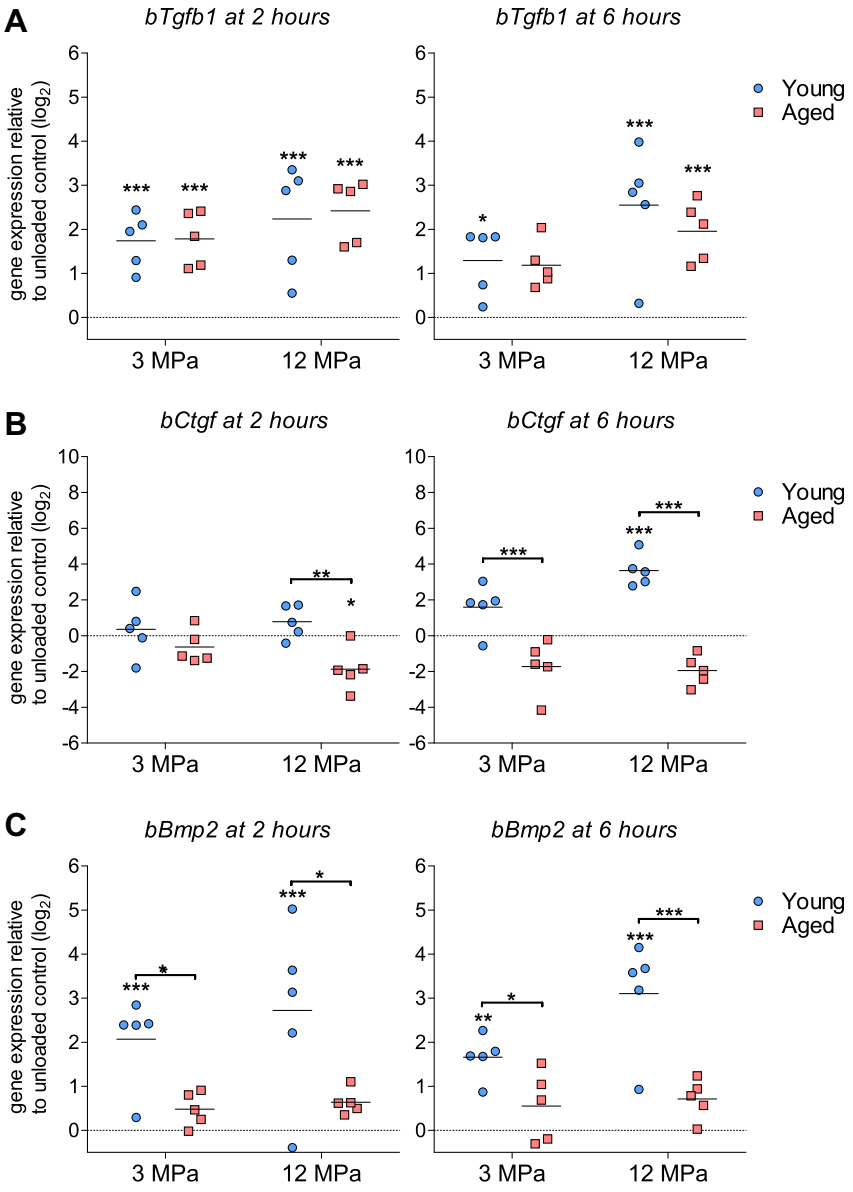

Fig. 4. Influence of dynamic mechanical compression on the expression of key tissue physiology mediators in cartilage of different age. The influence of 3 and 12 MPa dynamic mechanical compression on relative expression of (A) bTgfb1, (B) bCtgf and (D) bBmp2 in young and aged articular cartilage. Mechanical compression potently induced bTgfb1 expression in young and aged cartilage (A). However, mechanical compression had significantly different effect on bCtgf and bBmp2 expression regulation in cartilage of different age (B and C). Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal). Age of cartilage was as follows: Young-three 7 months, and two 11 months old, aged-12, 10, 8 and two 9 years old.*- $P \leq 0.05$; **- $P \leq 0.01$; ***- $P \leq 0.001$.

significant *bSerpine1* up-regulation was observed in young cartilage ($P = 0.003$ in 3 MPa and $P < 0.0001$ for 12 MPa compressed group) and in 12 MPa compressed aged cartilage ($P = 0.006$) (Fig. 6B). However, only in 12 MPa compressed cartilage, a significantly different levels of *bSerpine1* up-regulation levels between young and aged cartilage were observed (5.9-fold, $2^{2.6Ct}$ $P = 0.005$) (Fig. 6B) with higher expression levels in young cartilage.

Expression analysis of an alternative Smad3P responsive gene-*bJunB* confirmed the results of the *bSerpine1* measurements. Only in young cartilage, *bJunB* was greatly induced at 2 h after both levels of mechanical compression (22-fold $2^{4.5Ct}$ $P < 0.0001$ for 3 MPa and 25-fold $2^{4.7Ct}$ $P < 0.0001$ for 12 MPa) (Fig. 6C). At the same time in aged cartilage, *bJunB* expression showed no response to any level of compression (Fig. 6C). Therefore, *bJunB* expression responded significantly different to mechanical stimulus in young vs aged cartilage (17-fold, $2^{4.1Ct}$ $P < 0.0001$ for 3 MPa and 10-fold, $2^{3.3Ct}$ $P < 0.0001$ for 12 MPa stimulation group). At 6 h time point the age-related differences were not longer detectable.

Expression levels of another Smad3P responsive gene-*bSmad7* were analyzed but no age-related differences in regulation of *bSmad7* expression by mechanical compression were detected (Fig. 6D).

Discussion

Articular cartilage performs a very important biomechanical function being at the same time a highly mechanosensitive tissue. Cartilage experiences various forms of loads and these loads have been shown to play an important role in tissue formation, physiology and maintenance[1]. However, cartilage accumulates a number

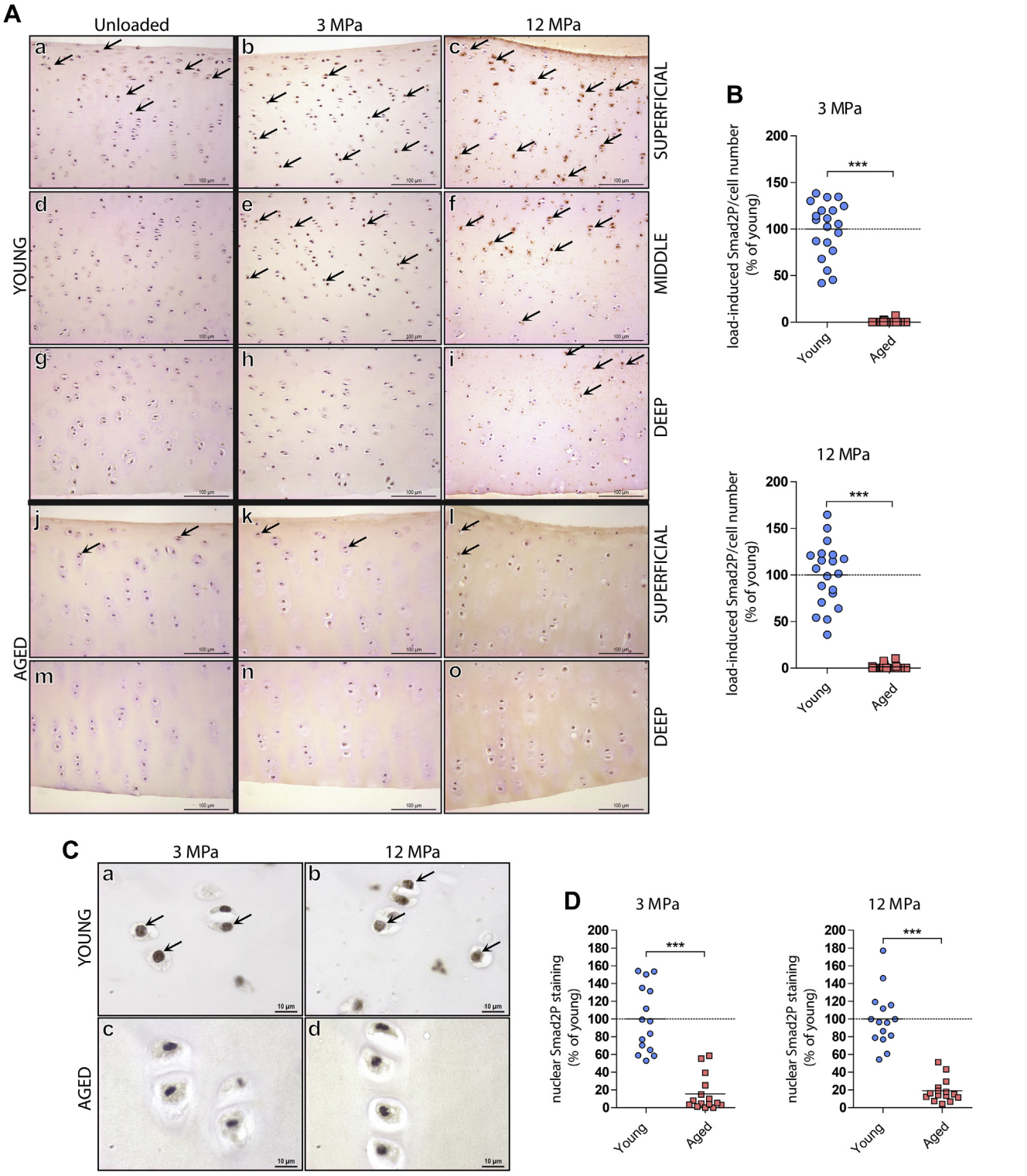

Fig. 5. Influence of dynamic mechanical compression on Smad2 activation in cartilage of different age. Clear induction of Smad2P staining was observed in young cartilage compressed with 3 and 12 MPa when compared to unloaded controls (Ab, e, h to Aa, d, g), (Ac, f, i to Aa, d, g). Clearly lower level of induction of Smad2P by compression was noticed in old cartilage compressed with 3 MPa and 12 MPa when compared to young cartilage (Ak, n to Ab, e, h) and (Al, o to Ac, f, i). Scoring of IH staining showed significantly lower load-induced Smad2P staining in aged 3 MPa and 12 MPa compressed cartilage when compared to young cartilage compressed with the same pressure (B). Smad2P staining in young compressed cartilage was most likely restricted to the cell nuclei (Ca, b) whereas in old compressed cartilage staining was more predominantly present in the cell cytoplasm (Cc, d). Scoring of nuclear IH staining showed significant reduction in Smad2P nuclear staining in aged cartilage when compared to young cartilage (D). Arrows indicate the Smad2P staining detected by threshold used for computational scoring of IH. Young cartilage-1 year old; aged cartilage-10 years old.

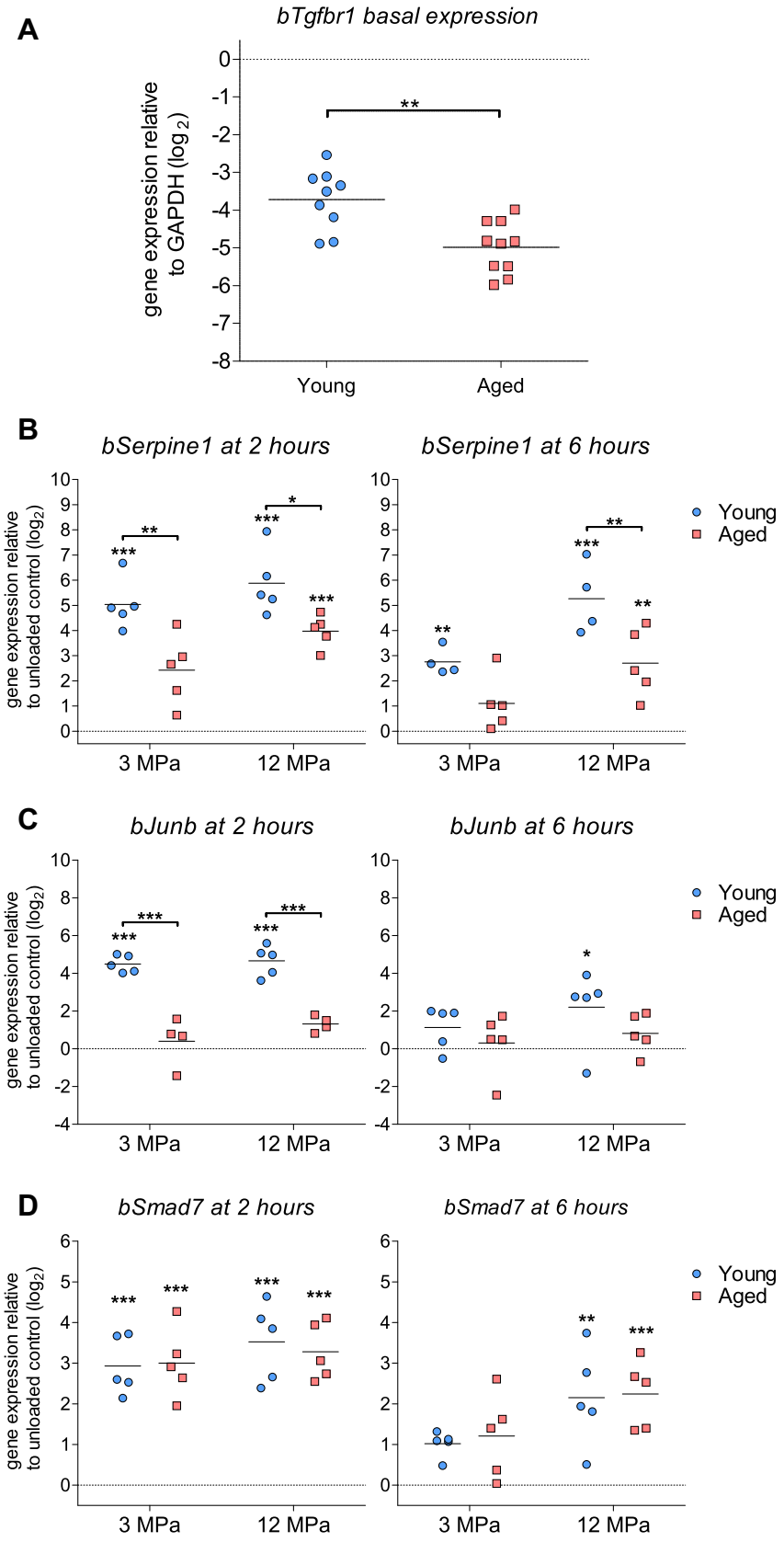

Fig. 6. Basal expression of Smad2/3 activating receptor (Tgfbr1) and influence of dynamic mechanical compression on relative expression of Smad3P reporter genes in cartilage of different age. (A) Basal expression of bTgfbr1 in cartilage of different age. The influence of 3 and 12 MPa dynamic mechanical compression on relative expression of (B) bSerpine1, (C) bJunB and (D) Smad7 in young and aged articular cartilage. Significantly higher bSerpine1 up-regulations were observed in young compressed cartilage than in aged (B). Moreover, only in young compressed cartilage the up-regulation of bJunB was detected (C). In both age groups, bSmad7 up-regulation was observed (D). Data are expressed as a grouped column scatter of multiple repeats with displayed mean (each point represents individual experimental repeat on material isolated from different animal). Age of cartilage was as follows: Young-three 7 months, and two 11 months old, aged-12, 10, 8 and two 9 years old.*- $P \leq 0.05$; **- $P \leq 0.01$; ***- $P \leq 0.001$.

of age-related changes in the ECM as well as in its cells[26]. Notwithstanding growing interest in the role of mechanical signals in cartilage biology, the knowledge on how age-related changes influence tissue mechanosensitivity is scarce. That is why the response of young and aged cartilage to physiological and excessive mechanical compression was investigated and compared. We report a reduced or loss of ability for regulation of various ECM components and essential tissue growth factors in aged cartilage by mechanical signals. However, most importantly, we identified a diminished ability for activation of Smad2/3 signaling as a response to mechanical load in aged cartilage. This indicates that age-related changes in articular cartilage have significant impact on the characteristic of tissue response to mechanical signals.

Previously our group has demonstrated that mature cartilage, when compressed, is activating Smad2/3 signaling which we hypothesized to be a consequence of latent TGFβ1 activation and subsequent signaling via TGFBR1 (ALK-5) receptor[6]. In the present study we show a highly reduced ability for mechanically mediated Smad2/3P signaling activation in aged cartilage. Moreover, we observed that Smad2P in young compressed cartilage is localized in cell nuclei whereas in aged cartilage is more predominantly present in the cytoplasm. This indicates that particularly in young dynamically compressed cartilage Smad2/3P was translocated to the nucleus to control transcription of genes. Not ruling out the role of other receptors known to be able to activate Smad2/3P signaling (ALK-4 and ALK-7) we think that this might be a consequence of age-related loss in bTGFBR1 (ALK-5) expression in articular cartilage which was shown in this study and also previously in murine cartilage[18]. The loss of ALK-5 receptor would disable the function of the mechanically activated TGFβ1 growth factor and subsequent Smad2/3P signaling activation. Moreover, a reduced synthesis of TGFβ ligands[21,27], could decrease the content of this ligands in aged cartilage ECM. This could negatively affect the extent of growth factor activation mediated by mechanical signals.

As previously reported, cartilage shows an age-related decrease in tensile fracture stress what indicates an alteration in biomechanical properties of this tissue when aged[28]. We show that higher pressure had to be applied on aged cartilage to achieve the same amount of deformation as in young cartilage. This indicates an increase in stiffness together with loss of water content in aged cartilage ECM. It is known that cells from many tissues are able to sense and respond to changes in ECM elasticity[29]. Because cartilage ECM is the major transducer of mechanical signals to the chondrocytes, increase in ECM stiffness could result in lower mechanosensitivity of aged cartilage and negatively affect mechanical signal transduction.

However, an alternative explanation for altered mechanically-mediated regulation of essential tissue growth factors and cartilage ECM components could be found in age-related changes in the expression of integrins[30] which are the main cell surface receptors transducing mechanical signals from the ECM and translating it to cell signaling pathways[31]. Because it has been shown that numerous of chondrocyte mechanosensitive signaling pathways are integrin-dependent[31], it can be speculated that alterations in the expression of integrins could have a significant impact on the mechanical signal transduction and further downstream gene expression regulated by load.

Numerous factors can control chondrocyte response to loads. Importantly, this also includes the cell's epigenetic status. Additionally, many studies reveal an epigenetic drift with aging also in cartilage[32]. This could indicate that in our experiments genes which are not responding to mechanical compression in aged cartilage compared to young cartilage, like *bBmp2*, are epigenetically repressed or silenced. Indeed an age-related epigenetically silencing in a promoter of another growth factor from the same family (BMP7) was found in cartilage[33]. Moreover, according to the Encyclopedia of DNA Elements data (ENCODE, http://genome.ucsc.edu/, release date of the genome assembly-20.01.2015), two CpG islands are present in the *Bmp2* promoter. It is known that DNA methylation, resulting in gene silencing, occurs mostly in CpG islands. Furthermore, ENCODE shows that several chromatin modifiers bind to the *Bmp2* promoter, including Histone-lysine N-methyltransferase (EZH2), which also in cartilage is involved in silencing of gene expression[34]. Independently, it has been reported that direct modulation of histone deacetylase (HDAC) activity, so the modulation of cell epigenetic status, can interfere with mechanically mediated gene expression[35]. Remarkably not all key growth factors genes were less responsive for mechanical signals in aged cartilage. The expression of *bTgfb1* gene was as potently induced in aged cartilage as in young. This shows that aged cartilage is not less responsive in all studied aspects and there are pathways induced by mechanical signals which are not altered by ageing.

There are some limitations of our study. First of all, as we showed, aged cartilage has highly reduced thickness when compared to young cartilage. By this, during unconfined compression of cartilage explant the force could possibly be differently distributed in samples with different thickness. However, our experiments were performed with force controlled set up, so there was always the same amount of force applied on the explant, not depending on specimen thickness. Moreover, age-related decrease of cartilage thickness appear also *in vivo*[26], but aged cartilage will in general experience a similar magnitude of loading like young. Secondly, our conclusions are based on a bovine animal model and might not be directly applicable to humans. However, as we demonstrated, bovine cartilage shows age-related changes in tissue gross appearance similar to changes observed in humans[26].

There are numerous studies showing the importance of mechanical signals in articular cartilage homeostasis. There are also many studies demonstrating age-related changes in the articular cartilage cells and ECM. Nevertheless, to our knowledge this is the first report connecting these two aspects and showing that aged cartilage responds differently to mechanical load compared to young cartilage. Here we report a disruption in Smad2/3 phosphorylation in aged articular cartilage, a pathway which is known to be protective for articular cartilage[20]. Our results demonstrate that the age of the articular cartilage greatly affects the response of tissue to mechanical signals. Age-related alteration in cartilage mechanotransduction can point to mechanisms of age-related articular cartilage diseases like OA.

Author contributions

Conception and design: Wojciech Madej, Pieter Buma, Peter van der Kraan.

Collection and assembly of data: Wojciech Madej, Arjan van Caam.

Analysis and interpretation of data: Wojciech Madej, Arjan van Caam, Esmeralda Blaney Davidson, Gerjon Hannink, Pieter Buma, Peter van der Kraan.

Drafting of the manuscript: Wojciech Madej, Pieter Buma, Peter van der Kraan.

Critical revision: Wojciech Madej, Arjan van Caam, Gerjon Hannink, Esmeralda Blaney Davidson, Pieter Buma, Peter van der Kraan.

Final approval of the article: Wojciech Madej, Arjan van Caam, Esmeralda Blaney Davidson, Gerjon Hannink, Pieter Buma, Peter van der Kraan.

Conflict of interest

The authors have no conflict of interest.

Acknowledgments

This study was supported by a grant from the Dutch Arthritis Association (grant LLP-15).

Supplementary materials

Supplementary materials related to this article can be found at http://dx.doi.org/10.1016/j.joca.2015.07.018.

References

1. Responte DJ, Lee JK, Hu JC, Athanasiou KA. Biomechanics-driven chondrogenesis: from embryo to adult. FASEB J 2012;26:3614–24.
2. Millward-Sadler SJ, Wright MO, Davies LW, Nuki G, Salter DM. Mechanotransduction via integrins and interleukin-4 results in altered aggrecan and matrix metalloproteinase 3 gene expression in normal, but not osteoarthritic, human articular chondrocytes. Arthritis Rheum 2000;43:2091–9.
3. Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, *et al*. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure *in vitro*. J Orthop Res 2003;21:110–6.
4. Wong M, Siegrist M, Cao X. Cyclic compression of articular cartilage explants is associated with progressive consolidation and altered expression pattern of extracellular matrix proteins. Matrix Biol 1999;18:391–9.
5. Vincent TL, McLean CJ, Full LE, Peston D, Saklatvala J. FGF-2 is bound to perlecan in the pericellular matrix of articular cartilage, where it acts as a chondrocyte mechanotransducer. Osteoarthritis Cartilage 2007;15:752–63.
6. Madej W, van Caam A, Blaney Davidson EN, van der Kraan PM, Buma P. Physiological and excessive mechanical compression of articular cartilage activates Smad2/3P signaling. Osteoarthritis Cartilage 2014;22:1018–25.
7. Furumatsu T, Matsumoto E, Kanazawa T, Fujii M, Lu Z, Kajiki R, *et al*. Tensile strain increases expression of CCN2 and COL2A1 by activating TGF-beta-Smad2/3 pathway in chondrocytic cells. J Biomech 2013;46:1508–15.
8. Nam J, Perera P, Rath B, Agarwal S. Dynamic regulation of bone morphogenetic proteins in engineered osteochondral constructs by biomechanical stimulation. Tissue Eng Part A 2013;19:783–92.
9. Lee HS, Millward-Sadler SJ, Wright MO, Nuki G, Salter DM. Integrin and mechanosensitive ion channel-dependent tyrosine phosphorylation of focal adhesion proteins and beta-catenin in human articular chondrocytes after mechanical stimulation. J Bone Min Res 2000;15:1501–9.
10. Li KW, Wang AS, Sah RL. Microenvironment regulation of extracellular signal-regulated kinase activity in chondrocytes: effects of culture configuration, interleukin-1, and compressive stress. Arthritis Rheum 2003;48:689–99.
11. Wells T, Davidson C, Morgelin M, Bird JL, Bayliss MT, Dudhia J. Age-related changes in the composition, the molecular stoichiometry and the stability of proteoglycan aggregates extracted from human articular cartilage. Biochem J 2003;370:69–79.
12. Bayliss MT, Osborne D, Woodhouse S, Davidson C. Sulfation of chondroitin sulfate in human articular cartilage. The effect of age, topographical position, and zone of cartilage on tissue composition. J Biol Chem 1999;274:15892–900.
13. DeGroot J, Verzijl N, Wenting-van Wijk MJ, Jacobs KM, Van El B, Van Roermund PM, *et al*. Accumulation of advanced glycation end products as a molecular mechanism for aging as a risk factor in osteoarthritis. Arthritis Rheum 2004;50:1207–15.
14. Chen AC, Temple MM, Ng DM, Verzijl N, DeGroot J, TeKoppele JM, *et al*. Induction of advanced glycation end products and alterations of the tensile properties of articular cartilage. Arthritis Rheum 2002;46:3212–7.
15. Martin JA, Buckwalter JA. The role of chondrocyte senescence in the pathogenesis of osteoarthritis and in limiting cartilage repair. J Bone Joint Surg Am 2003;85-A(Suppl 2):106–10.
16. Loeser RF, Shanker G, Carlson CS, Gardin JF, Shelton BJ, Sonntag WE. Reduction in the chondrocyte response to insulin-like growth factor 1 in aging and osteoarthritis: studies in a non-human primate model of naturally occurring disease. Arthritis Rheum 2000;43:2110–20.
17. Chubinskaya S, Kumar B, Merrihew C, Heretis K, Rueger DC, Kuettner KE. Age-related changes in cartilage endogenous osteogenic protein-1 (OP-1). Biochim Biophys Acta 2002;1588:126–34.
18. Blaney Davidson EN, Remst DF, Vitters EL, van Beuningen HM, Blom AB, Goumans MJ, *et al*. Increase in ALK1/ALK5 ratio as a cause for elevated MMP-13 expression in osteoarthritis in humans and mice. J Immunol 2009;182:7937–45.
19. van der Kraan PM, Blaney Davidson EN, van den Berg WB. A role for age-related changes in TGFbeta signaling in aberrant chondrocyte differentiation and osteoarthritis. Arthritis Res Ther 2010;12:201.
20. Yang X, Chen L, Xu X, Li C, Huang C, Deng CX. TGF-beta/Smad3 signals repress chondrocyte hypertrophic differentiation and are required for maintaining articular cartilage. J Cell Biol 2001;153:35–46.
21. Blaney Davidson EN, Scharstuhl A, Vitters EL, van der Kraan PM, van den Berg WB. Reduced transforming growth factor-beta signaling in cartilage of old mice: role in impaired repair capacity. Arthritis Res Ther 2005;7:R1338–47.
22. Mow VC, Wang CC, Hung CT. The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. Osteoarthritis Cartilage 1999;7:41–58.
23. Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier JM. Direct binding of Smad3 and Smad4 to critical TGF beta-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. EMBO J 1998;17:3091–100.
24. Jonk LJ, Itoh S, Heldin CH, ten Dijke P, Kruijer W. Identification and functional characterization of a Smad binding element (SBE) in the JunB promoter that acts as a transforming growth factor-beta, activin, and bone morphogenetic protein-inducible enhancer. J Biol Chem 1998;273:21145–52.
25. Nagarajan RP, Zhang J, Li W, Chen Y. Regulation of Smad7 promoter by direct association with Smad3 and Smad4. J Biol Chem 1999;274:33412–8.
26. Lotz M, Loeser RF. Effects of aging on articular cartilage homeostasis. Bone 2012;51:241–8.
27. Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan MF, Ferguson CM, *et al*. Microarray analysis reveals age-related differences in gene expression during the development of osteoarthritis in mice. Arthritis Rheum 2012;64:705–17.
28. Kempson GE. Age-related changes in the tensile properties of human articular cartilage: a comparative study between the femoral head of the hip joint and the talus of the ankle joint. Biochim Biophys Acta 1991;1075:223–30.

29. Discher DE, Janmey P, Wang YL. Tissue cells feel and respond to the stiffness of their substrate. Science 2005;310:1139–43.
30. Shakibaei M, Abou-Rebyeh H, Merker HJ. Integrins in ageing cartilage tissue *in vitro*. Histol Histopathol 1993;8:715–23.
31. Loeser RF. Integrins and chondrocyte-matrix interactions in articular cartilage. Matrix Biol 2014;39:11–6.
32. Barter MJ, Bui C, Young DA. Epigenetic mechanisms in cartilage and osteoarthritis: DNA methylation, histone modifications and microRNAs. Osteoarthritis Cartilage 2012;20:339–49.
33. Loeser RF, Im HJ, Richardson B, Lu Q, Chubinskaya S. Methylation of the OP-1 promoter: potential role in the age-related decline in OP-1 expression in cartilage. Osteoarthritis Cartilage 2009;17:513–7.
34. Schwarz D, Varum S, Zemke M, Scholer A, Baggiolini A, Draganova K, *et al*. Ezh2 is required for neural crest-derived cartilage and bone formation. Development 2014;141:867–77.
35. Saito T, Nishida K, Furumatsu T, Yoshida A, Ozawa M, Ozaki T. Histone deacetylase inhibitors suppress mechanical stress-induced expression of RUNX-2 and ADAMTS-5 through the inhibition of the MAPK signaling pathway in cultured human chondrocytes. Osteoarthritis Cartilage 2013;21:165–74.